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Abstract: Chloroform and methanol are superior solvents for lipid extraction from photosynthetic microorganisms, because they can overcome the resistance offered by the cell walls and membranes, but they are too toxic and expensive to use for large-scale fuel production. Biomass from the photosynthetic microalga Scenedesmus, subjected to a commercially available pre-treatment technology called Focused-Pulsed® (FP), yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME) after extraction with a range of solvents. FP treatment increased the FAME-to-crude-lipid ratio for all solvents, which means that the extraction of non-lipid materials was minimized, while the FAME profile itself was unchanged compared to the control. FP treatment also made it possible to use only a small proportion of chloroform and methanol, along with isopropanol, to obtain equivalent yields of lipid and FAME as with 100% chloroform plus methanol.

Research Highlights

- Pulsed Electric field (PEF) pretreatment enhanced lipid recovery from *Scenedesmus.*
- Extraction of non-lipid materials minimized with PEF as evidenced by higher FAMEs.
- Pretreatment minimized toxic solvent usage by 12-fold.

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Effects of pulsed electric field treatment on enhancing lipid recovery from the microalga, Scenedesmus

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Abstract

Chloroform and methanol are superior solvents for lipid extraction from photosynthetic microorganisms, because they can overcome the resistance offered by the cell walls and membranes, but they are too toxic and expensive to use for large-scale fuel production. Biomass from the photosynthetic microalga Scenedesmus, subjected to a commercially available pre-treatment technology called Focused-Pulsed® (FP), yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME) after extraction with a range of solvents. FP treatment increased the FAME-to-crude-lipid ratio for all solvents, which means that the extraction of non-lipid materials was minimized, while the FAME profile itself was unchanged compared to the control. FP treatment also made it possible to use only a small proportion of chloroform and methanol, along with isopropanol, to obtain equivalent yields of lipid and FAME as with 100% chloroform plus methanol.

Introduction

Photosynthetic microorganisms, i.e., algae and cyanobacteria, are capable of generating lipids that can become feedstock for producing liquid fuels currently generated from petroleum (Rittmann, 2008; Chisti, Y., 2007). Several species of microalgae, including Scenedesmus, Chlorella, Nannochloropsis, and Chlamydamonas, can fix carbon dioxide into high-density lipid inclusions that cause the microalgae to have 30-60% of their cell dry weight as lipids (Liang et al., 2009; Bondioli et al., 2012).

Lipids occur mainly as triacylglycerols (TAGs) in algae and diacylglycerols (DAGs) in cyanobacteria. TAGs are enclosed within intracellular oleosomes (Hu et al., 2008), and DAGs are contained in intracellular thylakoid membranes (Hu et al., 2008). Extraction of these intracellular lipids demands that the solvent be able to penetrate the cell wall and outer membranes, both of which may restrict its access (Sheng et al., 2011b; Zbinden et al., 2013; Goettel et al., 2013; Dejoye et al., 2011).

Two strategies have been evaluated to overcome resistance to solvent access: (1) extracting the lipids with very strong solvents that dissolve the lipids and break down the linkage between the lipids and membrane matrix, and (2) disrupting the cell's protective layers through pre-treatment so that accessibility is improved for any added solvent. The "gold standard" solvents are combinations of chloroform and methanol, such as Folch (1:1 chloroform: methanol) and Bligh & Dyer (B&D, 1:1:0.5 chloroform: methanol: water). While effective, lipid extraction with chloroform and methanol is infeasible for large-scale application, because these solvents are hazardous materials and expensive (Zbinden et al., 2013). Moreover, these strong

solvents co-extract non-lipid components from the biomass, necessitating extensive downstream refining of the valuable fuel precursors (Sheng et al, 2011a).

Recent approaches to make lipid recovery more sustainable include solvent-free extraction, such as supercritical CO_2, or "green" solvents, such as hexane, ethyl acetate, and isopropanol. While circumventing environmental toxicity, these approaches have achieved comparatively lower yields, although pre-treatment has been helpful (Dejoye et al, 2011; Zbinden et al., 2013; Sheng et al., 2011a; Bligh and Dyer, 1959; Folch, 1957).

Several pre-treatment techniques have been applied to improve lipid recovery through cell disruption and lysis. The goals are to make low-toxicity solvents work at least as well as the toxic solvents and to reduce the energy inputs for mixing and heating (Zbinden et al., 2013). Well-studied pre-treatment approaches for lipid extraction from photosynthetic biomass include mechanical, ultrasound, microwave, osmotic shock, enzymatic lysis, and pulsed electric fields (Sheng et al., 2011b; Zbinden et al., 2013; Goettel et al., 2013; Dejoye et al., 2011). The most recent entry applies a pulsed electric field (PEF) to disrupt biomass. This commercial technology is referred as Focused-Pulsed® (FP, OpenCEL, Atlanta, GA, http://www.opencel.com), and it has been documented to enhance hydrolysis and bioavailability for a range of biomass sources (Rittmann, 2008; Salerno et al., 2009). When FP is applied to disrupt biomass passed through a high-strength electrical field (> 30 kV) that is pulsed (~ 2000 hz), it disrupts cell membranes and walls as the electrical field interacts with phospholipids and the peptidoglycan.

Initial trials with PEF treatment of cyanobacteria and microalgae demonstrated enhanced lipid recovery (Sheng et al., 2011b, Zbinden et al., 2013; Goettel et al.,

2013), but solvent extraction remained the rate-limiting step. Here, a systematic study of how FP treatment disrupts Scenedesmus documents how disruption makes it possible to diminish significantly the use of toxic solvents without compromising lipid recovery in the form of FAMEs.

Materials and methods

Sample procurement

40 L of freshly harvested Scenedesmus spp. was obtained from a pilot-scale photobioreactor at the Arizona Center for Algal Technology and Innovation (AzCATi) located at ASU's Polytechnic campus. The Scenedesmus had been grown under nutrient-depleted conditions for achieving high lipid content (Hu et al, 2008). After transport to the Swette Center for Environmental Biotechnology (SCEB) on ASU's Tempe campus, the sample was subjected to FP treatment with the alpha unit at a treatment intensity of 30.6 KWh/m^3; this is called 1-pass treatment (Salerno et al, 2009). A portion of the sample that was collected from AzCATi was not subjected to treatment and was the control sample. The treated biomass (stored overnight at 4°C) was again passed through the FP unit to achieve 2-pass treatment. The second treatment achieved a treatment intensity of 33.7 KWh/m^3. Overnight cooling prior to the second pass ensured that cell lysis was not caused by a temperature increase. The temperature increased from 24°C to 54 °C after 1-pass treatment, while 2-pass treatment increased the temperature from 13.5°C to 36°C. Dry weight was measured as total suspended solids (TSS), and the organic fraction of the dry weight was assayed as volatile suspended solids (VSS) according to Standard Methods (Rice et al, 2012). Total and semi-soluble chemical oxygen demand (TCOD and ssCOD) was assayed using HACH kits and quantification by absorbance at a wavelength of 620

nm. Semi-soluble COD was obtained after filtering the sample through a 1.2-µm glass filter (Salerno et al. 2009).

Flow Cytometry

Flow cytometry measurement (FCM) of SYTOX Green-stained samples was performed using a BD FACSAria (BD Biosciences, CA, USA) flow cytometer. When cell walls were compromised by FP, SYTOX molecules were able to penetrate the cells and exhibit their characteristic green fluorescence upon staining the DNAs. The SYTOX was applied according to manufacturer guidelines (Invitrogen, Carlsbad, CA). Excitation was with an air-cooled 20 mW argon ion laser at 488 nm, and the fluorescence emission of SYTOX was detected using a 510-550 nm FITC filter with readings counted for 10,000 events from each sample. The percentages of total SYTOX stained cells were reported in Table 1, which corresponds to green fluorescent (dead/ inactivated) cells.

Crude Lipids and FAME extraction by standard solvent mixtures

About 15 g (dry weight) of control and FP-treated Scenedesmus biomass was freeze-dried using a FreeZone Benchtop instrument (Labconco, MO, USA). Lipid extraction followed the protocol of Sheng et al. (2011a). The solvents were Bligh and Dyer (chloroform: methanol: water = 1:2:0.8, v/v), Folch (chloroform: methanol = 2:1, V/V), hexane, and isopropanol. The solvent-to-biomass ratio was 1: 5 (v/w) for all the methods, all extractions were carried out twice, and all analyses were performed in duplicate. The mixtures were vortexed for 3 hours using a vortex mixer (Scientific Industries, NY, USA) at room temperature. After the sample was filtered through a 0.2-µm PVDF membrane (Pall Science, NY, USA) to remove the biomass debris, the

crude lipids were dried in the filtrate in a Nitrogen evaporator (Labconco RapVap, MO, USA). The crude lipid weight was obtained by subtracting the total dried weight from the weight of the empty tubes and the weight of any breakthrough materials released from the syringe filter when the solvents alone were passed through. The statistical differences of crude-lipid and FAME recovery between control and FP pre-treatment were evaluated using the Independent-Samples t-test by SPSS 22 (IBM, Armonk, New York) for the cases of different solvents, solvent mixtures, and kinetic extraction.

Trans-esterification of dried crude lipid was performed by adding 2 ml of 3-N methanolic HCl (Sigma-Aldrich, MO, USA) to the entire dried lipid in a test tube and incubated the mixture at 85 ^{0}C in the oven for 2.5 h (Sheng et al., 2011a). For direct trans-esterification, 2 mL of 3-N methanolic HCl was added to 15 mg of freeze dried biomass in a test tube and incubating the mixture under similar conditions as for regular trans-esterification. After cooling the mixture to room temperature, 0.5 ml DI water and 1.55 ml hexane were added, the mixture was vortexed to extract the FAME components, and then the 1.5-ml volumes of hexane were pooled for FAME analysis. The FAME components were quantified using a gas chromatograph (Shimadzu GC 2010, Japan) equipped with a Supelco SP-2380 capillary column (30 m x 0.25 mm x 0.20 μm) and flame ionization detector (FID). The outputs were calibrated against a 37-Component FAME Mix standard (Supelco, PA, USA).

Crude Lipids and FAME extraction with solvent mixtures

Different volume ratios of the Folch solvent and isopropanol were tested on the same samples of control and FP-treated biomass. Maintaining the total solvent volume at 3 mL, the Folch: Isopropanol (% by volume) ratio was varied as follows: 0, 3.3, 8.3,

16.7, 33.3, 66.7 and 100%. The extraction performance at each ratio was evaluated in terms of crude lipids and FAME content. The crude lipid weight was obtained following the method mentioned above.

Effect of vortex time on crude lipids and FAMEs extraction efficiency

The effect of vortexing time as a measure of the energy input needed to achieve a target extraction efficiency was evaluated. Extraction efficiency for control- and FP-treated biomass was evaluated with vortexing times of 0.5, 1, 2, and 4 minutes, after which crude lipids and FAME contents were evaluated using extraction with 100% Folch solvent.

Results and discussion

Sample characterization before and after FP treatment

Table 1 summarizes how FP treatment affected key physical and chemical characteristic of Scenedesmus biomass. TSS and VSS were almost unchanged by FP-treatment; this is consistent with past work on other types of biomass (Sheng et al., 2011b; Salerno et al, 2009) and underscores that FP treatment disrupts the biomass instead of destroying it. One-pass FP treatment increased the concentration of ssCOD by 54%, but the second pass increased ssCOD by only another 9% (data not shown). The increases to ssCOD were substantially larger than for Synechocystis PCC 6803 cells for similar treatment intensity (Sheng et al., 2011b), which was only 5%. The pH decreased after FP treatment, probably due to the release of soluble fatty acids (Chen et al., 2012).

Flow Cytometry

Flow cytometry with the SYTOX stain gauged the efficiency of cell lysis by FP pretreatment. The green fluorescence intensity increased by several orders of magnitude for 1-Pass (from up to 10_3 to10_5 units). In addition, the fraction of stained (inactive) cells increased dramatically after FP treatment: from 5% in the control to 97% (as shown in Table 1).

Lipid and FAME recovery

Figure 1 shows that the lipid recovery associated with FP treatment and different solvents. Compared with control biomass, FP treatment improved crude-lipid recovery by about 47, 71, 78, and 90% for B&D, Folch, hexane, and isopropanol, respectively. The solvent-extraction performance followed a similar order similar to what has been reported in the literature: Folch > B&D >> hexane ≥ isopropanol (Sheng et al., 2011a; Keris-Sen et al., 2014). The impact of FP treatment was even greater for FAME: as much as a 310% increase for hexane.

FAME recovery was always lower than crude lipid recovery for all solvents, indicating co-extraction of non-lipid components, like protein, carbohydrate, and pigment (Laurens et al., 2012). Several combinations of isopropanol and Folch solvents following FP treatment yielded the maximum FAME-to-biomass ratio, around 21% (Fig 1b). FP treatment improved accessibility of these solvents to the FAME targets rather than non-FAME materials, and the FAME: crude lipid ratio increased. In addition, direct transesterification of the untreated biomass yielded total FAME of 21.5 3.4%, implying that FP treatment with the best combinations of solvent extraction could achieve ~100% of the maximum extractable FAME.

Solvent requirement reduced by FP treatment

The Folch solvent plays an important role in solubilizing lipids and liberating the bound lipids from the membrane matrix. Figure 2 shows that extracted crude lipids and FAME increased with an increasing volume ratio of Folch solvent in Folch + isopropanol mixtures. A clear advantage of using FP treatment is that it reduced the amount of Folch solvent needed to obtain an equivalent FAME yield. For FP-treated biomass, the FAME yield obtained by adding 66.7% Folch was similar to the FAME yield obtained by extraction with 100% Folch. Even more importantly, the FAME yield obtained from FP-treated biomass using only 8.3% Folch was higher than the FAME yield obtained from control biomass by using 100% Folch solvent. Therefore, FP treatment significantly reduced the need for toxic Folch solvent (~12-fold) to get an equivalent yield of FAMEs from control Scenedesmus biomass. FAME profiles (%) were similar for all conditions, which confirm that FP treatment did not modify the inherent FAME composition and it mainly helped to improve the extraction efficiency. In fact, increasing Folch solvent with FP treatment diluted the benefit due to a decline in the FAME-to-crude lipids ratio. Thus, an optimum solvent dosage for FAME recovery after FP treatment was achieved.

In addition, Figure 3 shows that FP treatment reduced the vortex time by almost two orders of magnitude to achieve the same recovery of crude lipids and FAME. FP-treated biomass gave nearly the same FAME-recovery efficiency after 2 minutes of vortex time as for the control after 3 hours of vortexing. Thus, FP treatment lowered the energy input needed for mixing.

Conclusions

FP treatment increased the yield of FAME by as much as 3.1-fold using hexane over control Scenedesmus, while also increasing the FAME-to-crude-lipid ratio for all

solvent conditions, and the FAME profile was not affected by FP treatment. Thus, extraction generated more of the truly useful fatty acids for biofuel production after the Scenedesmus biomass was treated by FP. FP treatment also reduced the usage of toxic solvents (chloroform and methanol) by 12-fold for equivalent yields of lipid and FAME and significantly lowered the mixing energy requirements. Thus, FP treatment provides a sustainable strategy for extracting fuel feedstock from photosynthetic microorganisms.

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Supporting Information

The supporting information contains 8 pages, with four sections: Transmission Electron Microscopy, Cell Lysis Evaluation by Flow Cytometer, Particle Size Analysis, FAME-to-crude lipid ratios, and the FAMEs profile for all solvents and pre-treatment conditions. This includes Figures S1 to S5 and Table S1.

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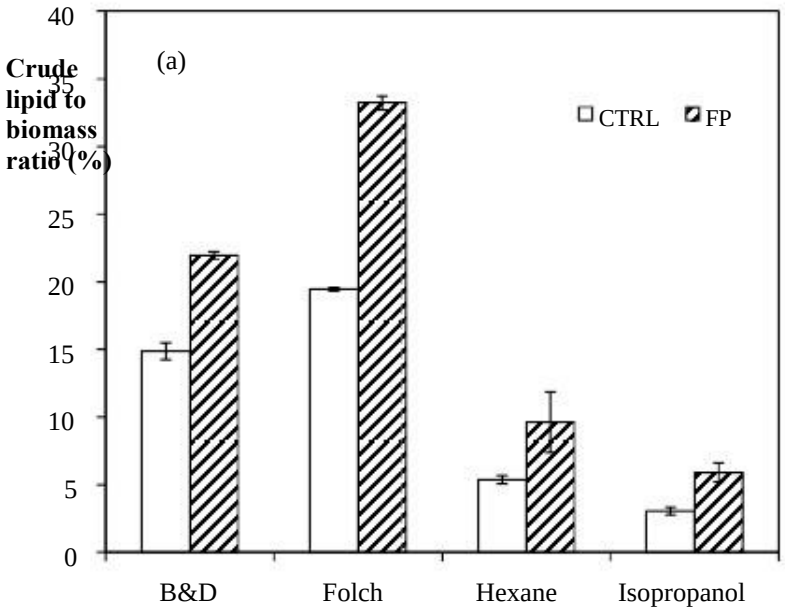
Table 1 Summary of physical and chemical parameters of Scenedesmus biomass before and after FP treatment

	Control	FP_1 pass
Treatment intensity (Kwh/m3)	--	30.6
Temperature change	24°C	26->53°C
pH	7.42	6.97
TSS (mg/L)	4600±40	4440±30
VSS (mg/L)	4470±50	4300±30
TCOD (mg/L)	8000±30	8000±60
ssCOD (mg/L)	450±10	690±10
Increased ssCOD (% to control)		50
% of total particles stained with SYTOX#	4.7	96.8

#10,000 cell counting events;



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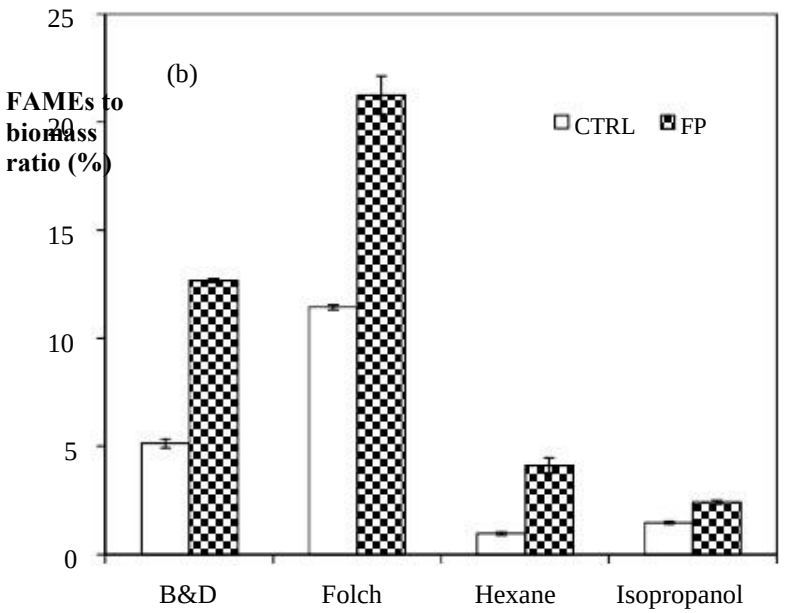

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318 Figure 1 Crude lipid (a) and FAME (b) recoveries (% of dry weight) for four solvent
319 systems -- Bligh and Dyer (B&D), Folch, hexane, and isopropanol -- for control and
320 FP-treated Scenedesmus biomass (1_pass) samples. Results for 2_pass samples were
321 similar and are not shown. The difference of FAME recovery was significant
322 between CTRL and FP within the group of the same solvent ($P < 0.05$).
323

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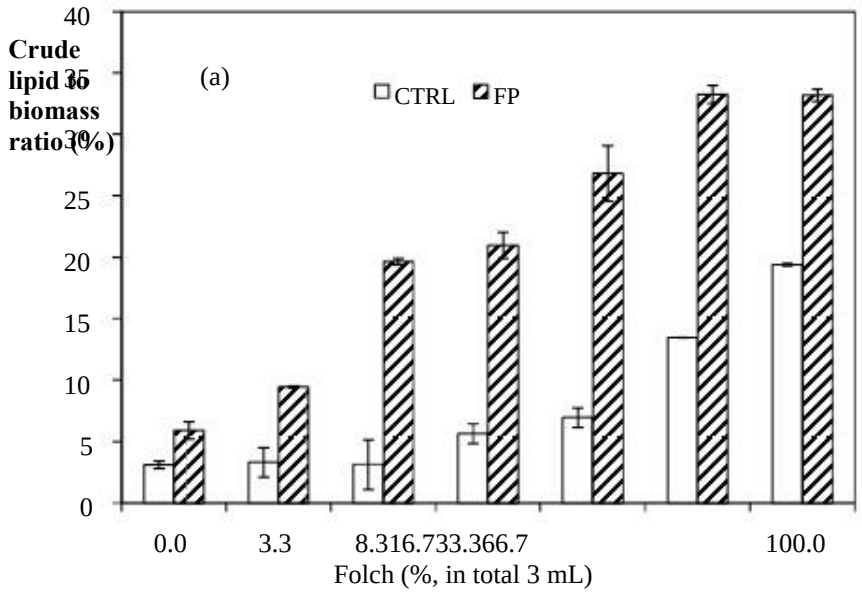

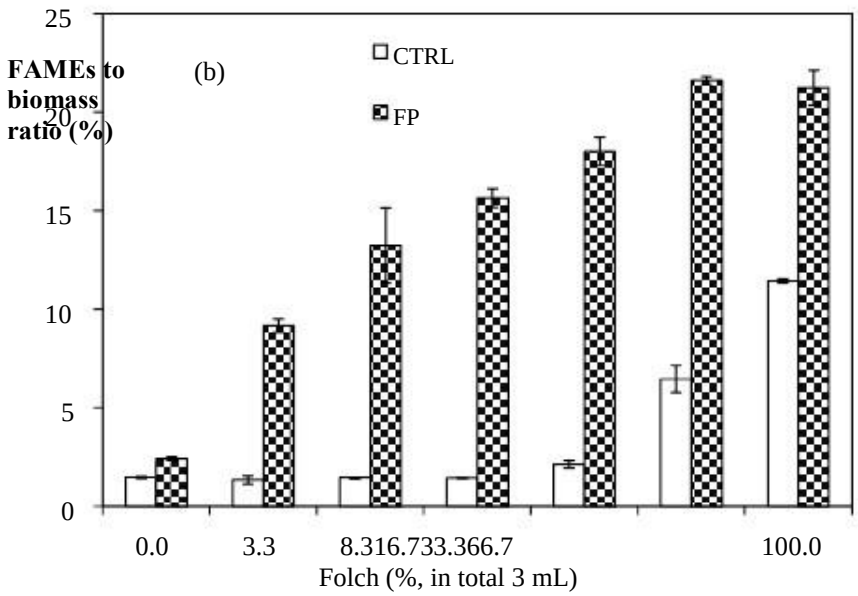

Figure 2 Crude lipid (a) and FAME (b) recoveries (% of dry weight) for different ratios of Folch and isopropanol solvent combinations with ratios (% by volume) for control and 1-pass FP-treated Scenedsmus biomass. The difference of FAME recovery was significant between CTRL and FP within the group of the same solvent ($P < 0.05$).

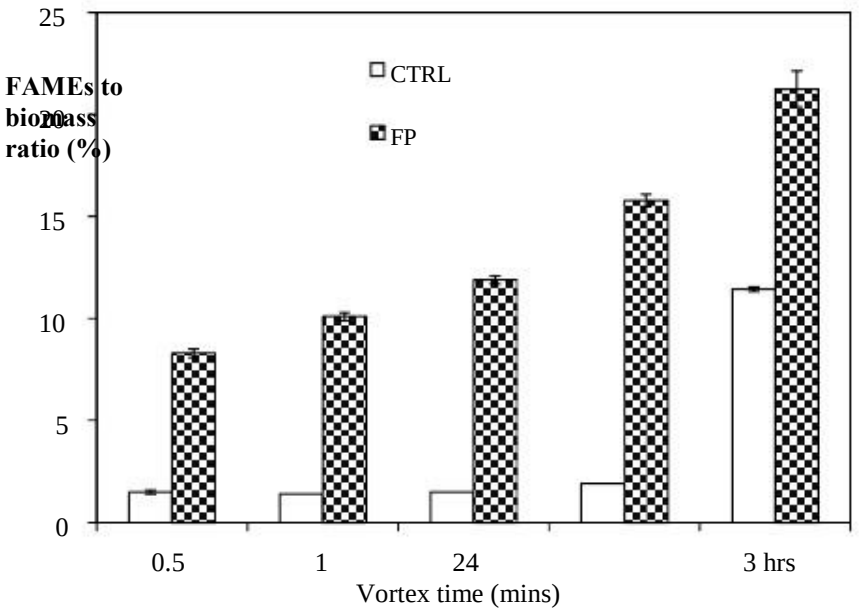

Figure 3 FAME recovery (% of dry weight) with different vortexing times for Control and 1-pass FP-treated Scenedesmus and using 100% Folch solvent. The difference of FAME recovery was significant between CTRL and FP within the same duration time of vortex ($P<0.05$).

Electronic Annex

Click here to download Electronic Annex: Supporting Information_Solvent Extraction_Re-revision 092214 final.docx